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PRINCIPAL INVESTIGATOR: David Gewirtz, Ph.D.

CONTRACTING ORGANIZATION: Virginia Commonwealth University
Richmond, VA 23298-0568

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These studies were designed to understand the role of c-*myc* and the p53 protein in the pathway leading to growth arrest in the breast tumor cell and in the relative refractoriness of breast tumor cells to the induction of apoptotic cell death. An additional component of this work was to investigate the repair of double-strand breaks induced by ionizing radiation (and by the radiomimetic, bleomycin) in breast tumor cells having wild-type versus mutant p53 genes and the relationship of double-strand break repair to apoptotic cell death. We have determined that radiosensitivity does not appear to be a function of p53 in breast tumor cells which fail to undergo apoptotic cell death and that suppression of c-*myc* may be a component of the signal transduction pathway leading to growth arrest in response to radiation only in p53 positive breast tumor cells. We have also determined that ionizing radiation fails to suppress the activity of the transcription factor E2F in breast tumor cells, suggesting the existence of a previously unidentified mechanisms for loss of restriction point control in the breast tumor cell. Finally, preliminary studies indicate that acquisition of a pleiotropic phenotype incorporating genomic instability and delayed reproductive death may be linked to mutagenesis induced by radiomimetic agents in 184B5 breast epithelial cells.

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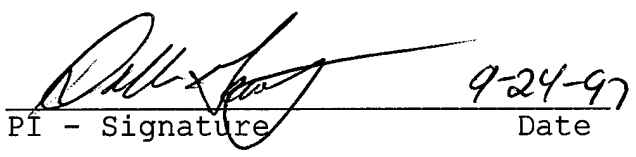

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INTRODUCTION *Subject and scope of research* This work is directed towards developing an understanding of the molecular and signal-transduction events mediating growth arrest and cell death in breast tumor cells after exposure to ionizing radiation. Our studies are focused primarily on two genes, p53 and myc, which appear to be instrumental in the regulation of cell growth and the response to DNA damage in breast tumor cells. We are also interested in the DNA damage response pathway which may link the response at the level of p53 to c-*myc* through intermediary genes in the DNA damage response pathway - i.e. by regulation of the cyclin dependent kinase inhibitory protein $p21^{waf1/cip1}$, dephosphorylation of the Rb protein and inhibition of the transcription factor, E2F. In addition, the capacity of breast tumor cells to recognize and repair (with fidelity) double-strand breaks is being investigated within the context of regulation of cell cycle progression by p53 and the induction of (apoptotic) cell death.

Background While ionizing radiation is one of the most effective treatments for breast cancer, its effectiveness is limited by the problem of tumor cell resistance. Some tumor cells may demonstrate primary resistance to radiotherapy, while others, though initially responsive, may acquire radioresistance during the course of treatment.

An extensive literature describes the signal transduction pathway which mediate growth arrest and/or cell death in irradiated cells. Irradiation, as well as other modalities which induce DNA damage are known to up-regulate levels of the tumor suppressor protein, p53 (Kuerbitz et al, 1992; Zhan et al, 1993; Dulic et al, 1994; Gudas et al, 1995), which in turn increases levels of the cyclin-dependent kinase inhibitory protein, $p21^{waf1/cip1}$ (Di Leonardo et al, 1994; Dulic et al, 1994; Bae et al, 1995; Gudas et al, 1995). Inhibition of cyclin dependent kinases results in abrogation of the phosphorylation of the tumor suppressor protein, Rb (Nigg et al, 1995; Dimri et al, 1996) - which then binds to and inactivates the transcription factor, E2F (Chellappan et al, 1991; Hiebert et al, 1992; Almasan et al, 1995). E2F is thought to regulate the expression of a spectrum of genes associated with DNA synthesis including c-*myc*, DNA polymerase alpha, thymidine kinase and thymidine synthetase (Almasan et al, 1995; Martin et al 1995). Interference with E2F function is postulated to block DNA synthesis and promote growth arrest (Johnson et al, 1993; Almasan et al, 1995). The p53, Myc and E2F proteins also have all been shown to mediate apoptosis or programmed cell death in a variety of tumor cell models in response to DNA damage (Evan et al, 1992; Almasan et al, 1995; Henneking et al, 1995; Lowe et al, 1995).

Although many types of DNA damage can cause an increase in p53 levels and activate the cascade described above, this pathway appears to be particularly sensitive to double strand breaks. Indeed, transfection experiments have suggested that the presence of one double-strand break in a cell nucleus, even on a nonessential plasmid, can activate a p53-dependent checkpoint and arrest the cell in G1 (Huang *et al.,* 1996). Thus, the upstream events in radiation-induced G1 arrest may be intimately linked to the recognition and processing of double-strand breaks.

Our interest in the signal transduction pathway involving p53, $p21^{waf1/cip1}$, the Rb tumor suppressor protein, E2F and c-*myc* is based on the following observations from our laboratories: 1)

the ionizing radiation has been found to induce damage preferentially in regions surrounding the c-*myc* gene (Bunch et al, 1995); 2) that the expression of c-*myc* was observed to decline in MCF-7 breast tumor cells exposed to ionizing radiation (Watson et al, 1997); and 3) MCF-7 cells were found to be refractive to apoptotic cell death after the induction of DNA damage (Fornari et al, 1996).

Purpose These studies were designed to understand the role of c-*myc* and the p53 protein in the pathway leading to growth arrest in the breast tumor cell and in the relative refractoriness of breast tumor cells to the induction of apoptotic cell death. An additional component of this work was to investigate the repair of double-strand breaks induced by ionizing radiation (and by the radiomimetic, bleomycin) in breast tumor cells having wild-type versus mutant p53 genes and the relationship of double-strand break repair to apoptotic cell death.

BODY

In the first specific aim, we proposed to test the hypothesis that ionizing radiation suppresses the expression of the oncogene, c-*myc* in select breast tumor cell lines and that radiation induced suppression of c-*myc* expression is a downstream event related to the induction of p53 and/or of $p21^{waf1/cip1}$.

In the second specific aim, we proposed to determine whether suppressed c-*myc* expression is required for growth arrest in breast tumor cells.

In the third specific aim, we proposed to assess the influence of ionizing radiation on the level, stability and activity of the Myc protein.

In the fourth specific aim, we proposed to test the hypothesis that suppression of c-*myc* expression and Myc protein activity is, in part, responsible for the relative refractoriness of breast tumor cells to apoptotic cell death.

A. c-*myc* expression, Myc protein levels and p53 status in sensitivity to ionizing radiation

Experimental Methods and Procedures Cell growth and radiation sensitivity were determined by trypan blue exclusion as well as clonogenic analyses. Effects of ionizing radiation on gene expression were assessed by Northern blotting; effects on protein levels were determined by immunoblotting. Apoptotic cell death was assessed based on alterations in cell morphology as well as by fluorescent end-labeling analyses.

Results and Discussion In comparing the influence of ionizing radiation on c-*myc* expression in MCF-7 cells (which are wild-type for p53) and MDA-MB231 cells (which have a mutated p53 gene) - we found that the suppression of c-*myc* expression in MCF-7 cells was time and dose-dependent (Figure 1), but that radiation produced a transient, dose-independent suppression of c-*myc* expression in MDA-MB231 cells (Figure 2). This finding suggested that functional p53 might be necessary for

the effects of radiation on c-*myc* expression. We further demonstrated that neither the MCF-7 nor the MDA-MB231 breast tumor cell lines underwent apoptotic cell death after irradiation. Figure 3 indicates that the extent of strand breaks determined by this assay was unaltered in irradiated cells. Both cell lines showed growth arrest alone, MCF-7 cells in G1 and G2, and MDA-MB231 cells exclusively in G2, consistent with the absence of functional p53 (Figure 4). Interestingly, there was no evident difference in radiosensitivity in these two cell lines (Figure 5), suggesting that the p53 status is not a primary factor in the radiation response.

Studies of the effects of ionizing radiation on Myc protein are currently in progress. However, we have preliminary data using another agent which suppresses c-*myc* expression in MCF-7 cells, the topoisomerase II inhibitor, VM-26, which suggest that Myc protein levels decline in concert with the decline in message expression. In addition, we have found that the decline in Myc protein levels is accompanied by a corresponding reduction in the activity of a Myc protein target, ornithine decarboxylase (Bello-Fernandez et al, 1993).

Conclusions and Implications We can draw the following conclusions from these studies.

1. Radiosensitivity in breast tumor cells does not appear to be a function of p53, at least in cells which fail to undergo apoptotic cell death.

2. Suppression of c-*myc* may be a component of the signal transduction pathway leading to growth arrest in response to radiation only in p53 positive breast tumor cells.

3. While both p53 and c-*myc* are clearly implicated in signal transduction pathways which respond to DNA damage, neither p53 nor c-*myc* is absolutely necessary for growth arrest in response to irradiation.

4. Preliminary findings that c-*myc* expression (and by extension, Myc protein levels) are not uniformly altered in different breast tumor cell lines should provide an indication of whether the Myc protein plays a role in apoptotic cell death in response to ionizing radiation.

B. p21 antisense studies to assess the effects of dysregulation of the cyclin-dependent kinase inhibitor p21 on the response of breast cancer cells to ionizing radiation.

Experimental Methods: MCF-7 cells have been transfected with a plasmid (pREP4; Invitrogen) containing the cDNA for the p21 coding region in the antisense configuration under the control of an RSV promoter as well as a hygromycin resistance gene. After electroporation, cells were maintained under selection pressure with 200 μg/ml hygromycin. Single cell cultures were then obtained by limiting dilution, and expanded in the continuous presence of hygromycin. Presently, clones derived from 30 single cell cultures have been isolated and are growing well in the presence of hygromycin, along with several clones containing only the empty vector. These clones are currently being expanded, and as soon as sufficient numbers of cells are obtained, they will be frozen and stored under liquid nitrogen.

Once cell numbers are sufficient, individual clones will be assayed for p21 response by monitoring p21 induction by Western analysis following exposure of cells to IR (6 Gy; 2-6 hr). Two-three clones will be selected which display the greatest inhibition of p21 induction compared to empty-vector controls. Once these clones are selected, their response to IR will be compared to controls with respect to (a) IR-induced cell death; (b) IR-mediated cell cycle perturbations; (c) down-stream G_1 arrest events, including pRb dephosphorylation, E2F complex formation alterations in c-*myc* protein expression, Myc protein levels and ornithine decarboxylase activity.

C. Role of the transcription factor E2F in the response of breast tumor cell to ionizing radiation.

Experimental Methods

In attempting to identify factors which regulate c-*myc* expression, we evaluated the influence of ionizing radiation on E2F activity in breast tumor cells by transiently transfecting the cells with an E2F binding site-luciferase reporter plasmid. Cells were irradiated and luciferase activity was assessed at various time intervals. All data were normalized for transfection efficiency by co-transfection with a plasmid expressing beta-galactosidase under control of a CMV promoter. We also assessed the influence of radiation on upstream effectors of E2F, p53, p21$^{wafl/cip[1}$ and the Rb protein by immunoblotting.

Results and Discussion.

Influence of ionizing radiation on the levels of p21 $^{wafl/cip1}$ and on the phosphorylation state of Rb

The induction of p21 $^{wafl/cip1}$ in response to DNA damage has been demonstrated to occur in cells exposed to ionizing radiation as well as to other modalities which induce DNA damage (Gudas et al, 1995; Dulic et al, 1994). Exposure of MCF-7 cells to 10 Gy of ionizing radiation resulted in a time-dependent increase in p21$^{wafl/cip1}$ levels (Figure 6, upper panel). The increase in p21 was roughly 2-fold after 3 hours; a 3-fold increase, which was evident by 6 hours was maintained over a period of 24 hours. These observations are similar to the findings of other investigators using a dose of 5 Gy in MCF-7 cells (Gudas et al, 1995).

The p21 $^{wafl/cip1}$ protein acts as a generalized inhibitor of the cyclin dependent kinases (Hiebert et al, 1992). One consequence of this inhibition is conversion of the phosphorylated form of the Rb tumor suppressor protein to the hypophosphorylated form (Weinberg, 1995), which is thought to activate Rb (Weinberg, 1995), and to facilitate its binding to the transcription factor E2F (Hiebert et al, 1992). The phosphorylation state of Rb in response to ionizing radiation was determined over the same time frame as the levels of the p21 $^{wafl/cip1}$ protein. Results of the Western analysis, presented in the lower panel of Figure 6, are similar to what has been reported by other investigators assessing the status of Rb in proliferating MCF-7 breast tumor cells (Kwon et al, 1996; Gorospe et al, 1996, Wosikowski et al, 1995); that is, in control cells (the first five lanes) we observe a broad band indicative of the multiple phosphorylated states of the Rb protein. Figure 6 (lower panel) indicates that the dephosphorylated form of Rb was discernible after 3 hours, and was further visible

throughout the 24 hour interval subsequent to irradiation. However, in these studies with ionizing radiation, the phosphorylated form of Rb was still evident even at 24 hours after irradiation (last lane). Furthermore, there was an indication of a decline in the overall levels of the Rb proteins at 24 hours. It should be noted that this decline is not a result of DNA fragmentation associated with apoptotic cell death.

Effects of ionizing radiation on E2F activity.

The drug and radiation induced increases in p21 $^{waf1/cip1}$ levels and the dephosphorylation of Rb should result in a concomitant inactivation of the transcription factor E2F (Hiebert et al, 1992; Dimri et al, 1996). The influence of ionizing radiation on E2F activity was assessed at intervals of 4 and 24 hours after irradiation with 6 Gy. Figure 7 indicates that there was no discernible diminution of E2F activity by ionizing radiation. Figure 7 also indicates that a reporter plasmid containing mutations in the E2F binding site (pGL2-E2F-mut/luc; see Materials and Methods) which was utilized as a negative control at 12 hours had relatively low expression of luciferase as compared to the wild-type plasmid containing intact E2F binding sites.

Determination of the functional interaction between Rb and E2F in the MCF-7 cell line

The absence of suppression of E2F activity by ionizing radiation in cells with elevated levels of the p21 $^{waf1/cip1}$ protein and dephosphorylation of Rb might be ascribed to problems with the reporter assay or to defects in the association between Rb and E2F. These issues were addressed by transfecting cells with vectors constitutively expressing exogenous Rb or the adenoviral protein, E1A, either alone or in combination. Transfection with Rb was designed to demonstrate that endogenous E2F activity could be inhibited by its binding to the Rb tumor suppressor protein. Transfection with E1A, which dissociates E2F complexed with the Rb family of proteins (Arroyo et al, 1992; Barbeau et al, 1994) was designed to further validate the utility of the reporter assay in detecting alterations in E2F activity. As expected in cells with functional E2F, the Rb expressing vector produced a marked reduction in E2F activity. The average reduction in E2F activity was 67.7 ± 16.5%. Conversely, transfection with E1A stimulated E2F activity (data not shown), indicating the dissociation of endogenous complexes involving E2F and the Rb family of proteins (i.e. p130, p107 and Rb) in MCF-7 cells. Transfection with Rb was able to reverse the stimulation of E2F activity induced by E1A, a finding which is consistent with the capacity of E2F to be bound to and inhibited by Rb.

Conclusions and Implications These observations indicate that c-*myc* expression does not appear to be regulated by E2F in response to DNA damaging agents. These studies further suggest that alterations in Rb phosphorylation are not predictive of E2F activity. The greatest potential significance of these findings is the possibility that the breast tumor cell is functionally defective in its capacity to regulate E2F activity in response to DNA damage. This could lead to an understanding of the basis for loss of restriction point control and development of breast cancer as a result of mutations/DNA damage at sites other than the most common mutation in human malignancies, the p53 gene.

D. Mutagenesis

In specific aim 5, we proposed to examine, at the DNA sequence level, double-strand break repair events in breast tumor cells treated with radiation and with the radiomimetic drug bleomycin, by analysis of deletions and rearrangements induced by these genes in the HPRT locus.

In specific aim 6, we proposed to compare double-strand break repair events in breast tumor cells having normal vs mutant p53 genes and, if possible, in breast tumor cells rendered competent for radiation-induced apoptosis.

Experimental Methods 184B5 cells were grown in 100-mm plastic Petri dishes, and usually subcultured at ~80% confluence. Medium used for routine culture (hereafter referred to as serum-containing medium) was a 1:1 mixture of Ham's F12 and Dulbecco's media, reconstituted from powder (Gibco), filter-sterilized, and supplemented with 0.5 μg/ml hydrocortisone, 20 ng/ml epidermal growth factor, 100 ng/ml cholera toxin, 25 μg/ml insulin, 5% horse serum, and antibiotics. Procedures requiring more stringently controlled growth or cloning of individual cells were performed using commercially prepared Mammary Epithelial Cell (MEC) medium from Clonetics Corp. This medium contains, in addition to the usual low-molecular-weight components, bovine pituitary extract, epidermal growth factor, insulin, hydrocortisone and antibiotics, but no serum.

For subculturing, cells were washed with PBS and detached with 0.25% trypsin in PBS plus 1 mM EDTA. An equal volume of serum-containing medium was added, and the cells were pelleted ($100 \times G$, 10 min) in order to remove trypsin. The cells were resuspended and seeded at a density of 10^5 per dish in the serum- containing medium.

For mutagenesis experiments, cells were grown to 80% confluence in MEC medium, and then the medium was replaced with the same medium but lacking epidermal growth factor. Beginning one day later, these G0 cells were treated with bleomycin (0.5 - 5 μg/ml) for two days, with both medium and drug being replaced after one day. Following the 2-day treatment, the drug was removed, the cells washed, and the medium again replaced with drug-free, growth factor-free medium. After 4 hr of recovery in this medium, the cells were trypsinized and plated at 800 cells per plate in MEC medium to determine survival. The bulk of the cells were seeded at 10^5 cells per plate in the serum-containing medium, with 10 plates for each treated culture. The cells usually reached confluence 7 days later, and at that time they were again trypsinized and plated in Clonetics medium containing 10 μM 6-thioguanine (5×10^4 cells per plate, 20 plates from each initial treated culture) in order to select mutants. After 8 days, colonies were counted and two mutant colonies from each initial culture were trypsinized inside a cloning ring. The trypsinized cells were seeded into 100-mm plates containing serum-containing medium. The cells were subcultured once and then trypsinized, washed, frozen in medium containing 10% DMSO, and stored in liquid nitrogen.

Results and Discussion

Selection of cell lines and media: Mutagenesis experiments required a cell line which (like

normal cells) is functionally hemizygous at the *HPRT* locus, and can form clones from single cells with high efficiency. It is preferable that the line have a stable karyotype, and since we intend to examine the effect of p53, the initial line should have normal p53 function. The ZR-75 and MCF7 lines already in use in the laboratory were found to be unsuitable, the former because it is tetraploid and the latter because *HPRT* genes on both X chromosomes are expressed in this line (Wang *et al.*, 1990).

Two new candidate lines, 184B5 and MCF10A, were therefore obtained and evaluated; both these lines are immortal but nontransformed and have stable, near-diploid karyotypes (Walen and Stampfer, 1989). MCF10A, however, was found to have poor clonogenicity at low plating density. In contrast, 184B5, which had been used in some earlier mutagenesis experiments (Eldridge and Gould, 1991), consistently had plating efficiencies in excess of 50%, and was chosen for pilot experiments. Fortuitously, several 184B5 derivative lines, in which p53 function has been disrupted by transfection of the human papilloma virus E6 protein, were isolated by Gudas *et al.*, (1995), and two such lines were obtained from these investigators.

Several serum-free and serum-containing media were tested for ability to support growth of the 184B5 cells. The only medium that would reproducibly support robust clonal growth was a defined, commercially prepared medium, available from Clonetics Corp. at $150 per liter. However, a second, serum-containing medium was found to support routine cell growth. Hence, we are currently using the Clonetics medium for plating out single colonies in mutation selection and survival assays, as well as for drug treatments (in order to avoid possible interference by serum). The serum-containing medium, which we can prepare for ~$40 per liter, is used for all other steps and for routine propagation of cells.

Although a radiation-induced increase in the expression of $p21^{waf1/cip1}$ had already been demonstrated in 184B5 cells by Gudas *et al.* (1995), it was necessary to confirm that this DNA damage response pathway was intact through the final endpoint of G1 arrest. The effects of radiation on cell cycle progression were therefore determined by microfluorometry. When log-phase 184B5 cultures were incubated for 19 hr in the presence of nocodazole (which prevents cells from entering G1), the fraction of cells in G1/G0 decreased from 87% to 12%, due to progression of G1 cells into S phase. However, in parallel cultures which were irradiated (600 rads) prior to nocodazole addition, the G1/G0 fraction dropped only to 31% after 19 hr (Fig 8). Thus, radiation partially blocked the progression of G1 phase cells into S, indicating that the p53-dependent G1 arrest pathway is intact in these cells.

In contrast, in the E6-transfected cells obtained from Gudas *et al.*, radiation did not have any effect on the nocodazole-induced decrease in G1/G0 cell fraction (data not shown). This result indicates that, as expected, E6 expression in these cells inactivated the p53-dependent G1 arrest pathway. Thus, in later experiments, it should be possible to accurately assess whether p53 function is required for any given effect, by comparing results for 184B5 cells to results for the E6-expressing derivative.

Bleomycin-induced cytotoxicity and mutagenesis in G0-phase 184B5 cells: In previous experiments with CHO cells, bleomycin induced detectable mutagenesis only in confluence-arrested (G0-phase) cells. It was thus necessary to first establish whether 184B5 cells could be arrested in G0 phase, and whether they could subsequently be induced to resume normal growth. It was found that when cells were grown to ~80% confluence in the Clonetics medium, and then the medium was replaced with otherwise identical medium lacking epidermal growth factor, within 24 hr 95% of the cells were in G1/G0 phase as determined by microfluorometry. Moreover, upon trypsinization and replating at 10^5 cells per plate, growth of the arrested cells quickly resumed, and the S-phase population increased to 17% within one day. These results suggested that the 184B5 cells would be suitable for studies of mutagenesis in G0 phase. Similar results were obtained with the 184B5-E6 cells, in which p53 has been inactivated.

In a preliminary experiment, clonogenic survival was measured following bleomycin treatment (Table 1). A moderately toxic dose of 2 μg/ml was chosen for the initial mutagenesis experiment. Table 2 shows survival and mutagenesis data from the first two experiments. Bleomycin treatment clearly increased the mutation frequency, to a maximum of approximately 6 $\times$ 10^{-5}. However, the spontaneous mutation frequency was somewhat variable, such that in the second experiment, bleomycin treatment increased the mutation frequency only twofold. Thus, about half the mutants isolated from bleomycin-treated cells may be of spontaneous origin, a factor which will complicate interpretation of the mutation spectrum. Hence, in current experiments we are attempting to reduce the mutation frequency in untreated cells by growing the cultures prior to drug treatment in medium containing hypoxanthine, aminopterin and thymidine (HAT), which will eliminate any preexisting *HPRT* mutants in the cultures.

Analysis of the mutant clones is in progress, beginning with assessment of the presence of *HPRT* mRNA by Northern blot. Unexpectedly, however, gross morphological differences have already been noted between mutants from treated and untreated cultures. Specifically, the mutants from the treated cells required about twice as long as the those from untreated cells to grow from an initial trypsinized colony to confluence in a 100-mm plate. This was apparently due in large part to the persistent presence of large numbers of dead or dying cells in the mutant clones derived from treated cells.

This behavior of the mutant cells is at least superficially similar to the "delayed reproductive death" phenomenon reported by Chang and Little (1991), wherein a small fraction of the clones surviving X-irradiation of CHO cells continue to show persistent, extensive cell death and lowered plating efficiency many generations after irradiation. It is also reminiscent of our own recent work with bleomycin-treated CHO cells, wherein there appeared to be a correlation between certain kinds of bleomycin-induced specific-locus mutations, and a global loss of chromosomal stability in the mutant clones. In the studies of Chang and Little (1992) with irradiated cells, it was found that progeny clones showing acquired genomic instability had a high probability of also showing delayed reproductive death, even though only a small fraction of progeny clones showed either of these properties. Thus, although still very preliminary, our initial data are consistent with the proposal that acquisition of a similar pleiotropic phenotype, incorporating genomic instability and delayed

reproductive death, may be linked to certain types of bleomycin-induced mutagenesis in 184B5 cells.

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Legends to Figures

Figure 1 Analysis of dose-dependent effects of ionizing radiation on *c-myc* expression in MCF-7 cells. Quantitative representation of pooled data (mean ± standard error) from 4 replicate experiments. *Inset*: Representative Northern analysis indicating the dose-dependent reduction in c-*myc* expression and the relatively stable expression of GAPDH at 3 hours. The radiation dose is indicated above each lane in the autoradiograph.

Figure 2 Influence of ionizing radiation on *c-myc* expression in MDA-MB231 cells at 1 hour. Quantitative representation of pooled data (mean ± standard error) from 3 replicate experiments.*Inset*: Representative Northern analysis indicating the *absence of* a dose-dependent reduction in c-*myc* expression and the corresponding expression of GAPDH. The radiation dose is indicated above each lane in the autoradiograph.

Figure 3 TUNEL assay for the induction of DNA damage by ionizing radiation in MCF-7 and MDA-MB231 breast tumor cells. MDA-MB231 and MCF-7 cells (both adherent and non-adherent) were isolated on microscope slides at the indicated times after irradiation, and DNA fragmentation was assessed by fluorescent end-labeling.
Upper Panel: Shown are control MCF-7 cells and MCF-7 cells at 48 hours after irradiation with doses of 10 and 20 Gy. *Lower Panel*: Shown are control MDA-MB231 cells and MDA-MB231 cells at 48 hours after irradiation with doses of 10 and 20 Gy.

Figure 4. Influence of ionizing radiation on cell cycle traverse. Cells were isolated for determination of cell cycle distribution at 24 hours after exposure to 6 Gy of ionizing radiation.

Figure 5 Influence of ionizing radiation on proliferation of MCF-7 and MDA-MB231 breast tumor cells. The two breast tumor cell lines were irradiated with various doses of irradiation (0.5 to 10 Gy) and growth inhibition was calculated based on the relative growth rates of control and irradiated cells after 24 hours - where growth of control cells is taken as 100%. Values represent means ± standard errors for 4 replicate experiments (MCF-7) and 3 replicate experiments (MDA-MB231).

Figure 6 Influence of ionizing radiation on the levels of the p21 $^{waf1/cip1}$ protein and Rb dephosphorylation in MCF-7 cells. Cells were exposed to 10 Gy of radiation, and protein was isolated at the indicated times. Upper Panels: An approximately 2-fold increase in p21$^{waf1/cip1}$ levels is observed by 3 hours and a 3-fold elevation is evident within 6 hours that is sustained over 24 hours. **Lower Panel:** Levels and phosphorylation state of Rb.The broad band is indicative of the multiple phosphorylated states of the Rb protein. The lower band is the hypophosphorylated form of Rb. Conversion to the dephosphorylated form of the Rb protein corresponds closely with the increase in levels of p21$^{waf1/cip1}$ i.e. the change is evident at 3 hours, more pronounced at 6 hours and maintained over a period of at least 24 hours.

Figure 7 Influence of ionizing radiation on E2F activity in MCF-7 breast tumor cells. An E2F-luciferase reporter plasmid (PGL2-E2F/luc) which permits assessment of the activity of the multiple forms of E2F was transfected into MCF-7 cells along with a beta galactosidase expression vector to control for transfection efficiency. E2F activity was assessed at intervals of 4 and 24 hours after irradiation with 6 Gy. There was no discernible diminution of E2F activity. The reporter plasmid containing mutations in the E2F binding site was utilized as a negative control at 0 and 24 hours.

Figure 8 The effects of radiation on cell cycle progression in 184B5 cells. Cell cultures in log phase were incubated for 19 hr in the absence (top panel) or in the presence (middle panel) of nocodazole, or were first irradiated (600 rads) and then incubated in the presence of nocodazole for 19 hr (bottom panel). The fraction of cells in G1/G0, S and G2M was determined by microflow cytometry. The nocodazole-induced depletion of the G1/G0 fraction was partially abrogated by prior irradiation, indicating a radiation-induced G1 arrest.

Table 1: Survival of Cells Treated with Bleomycin at Different Concentrations

Bleomycin (μg/ml)	Number of Colonies	Survival (%)
5	4	2.19
2	35	19.23
0.25	191	104
0	182	100

Table 2: Mutagenesis in Cells Treated with Bleomycin

First time experiment:

Bleomycin (μg/ml)	Survival (%)	Frequency Mutation
0	100	8×10^{-6}
2	36	6×10^{-5}

Second time experiment:

bleomycin(μg/ml)	survival(%)	Mutation frequency
0	100	3.11×10^{-5}
2	40.36	7.35×10^{-5}
4	23.49	5.58×10^{-5}

Figure 1

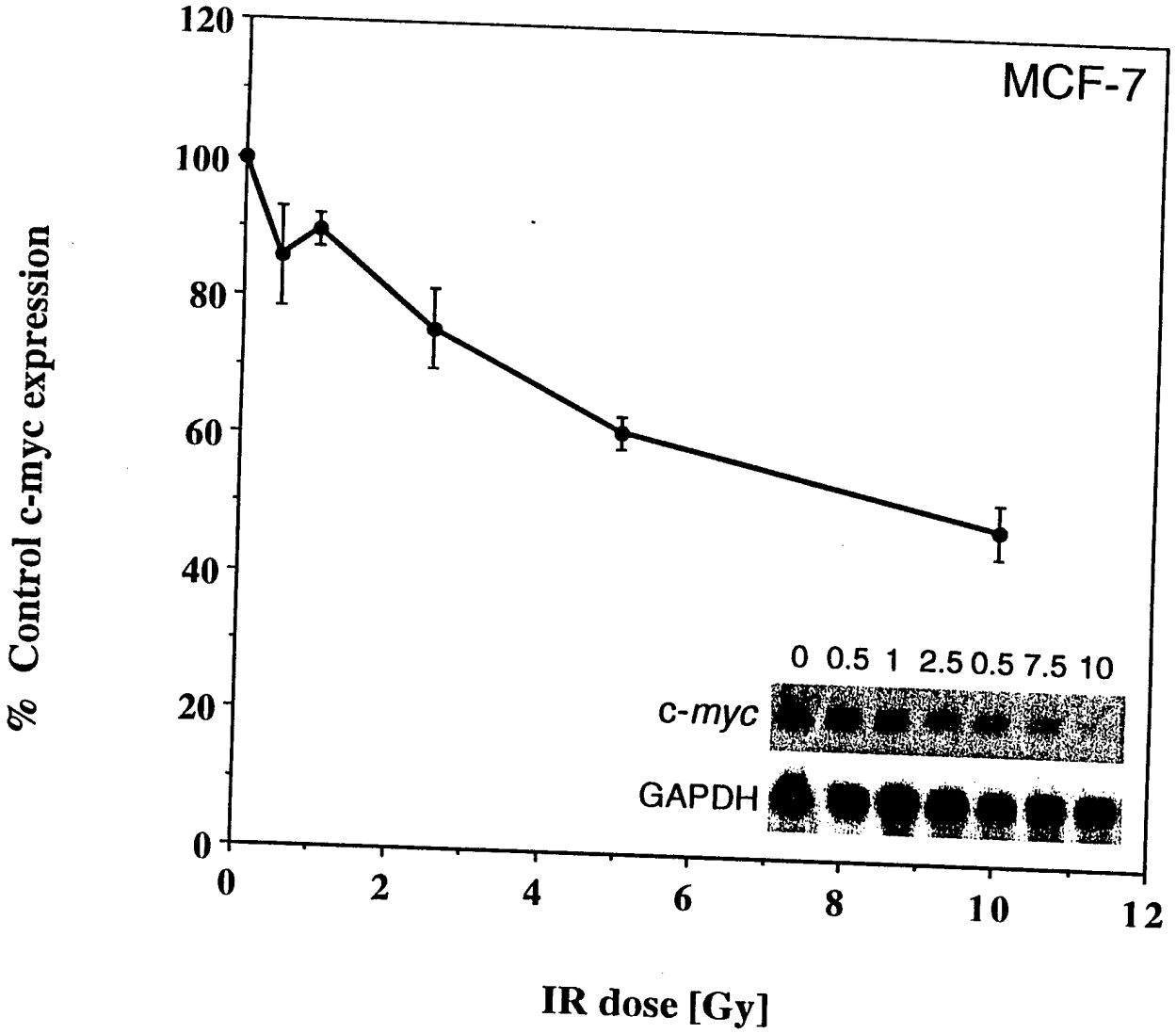

MCF-7
% Control c-myc expression
IR dose [Gy]
0 0.5 1 2.5 0.5 7.5 10
c-myc
GAPDH
120
100
80
60
40
20
0
0
2
4
6
8
10
12

Figure 2

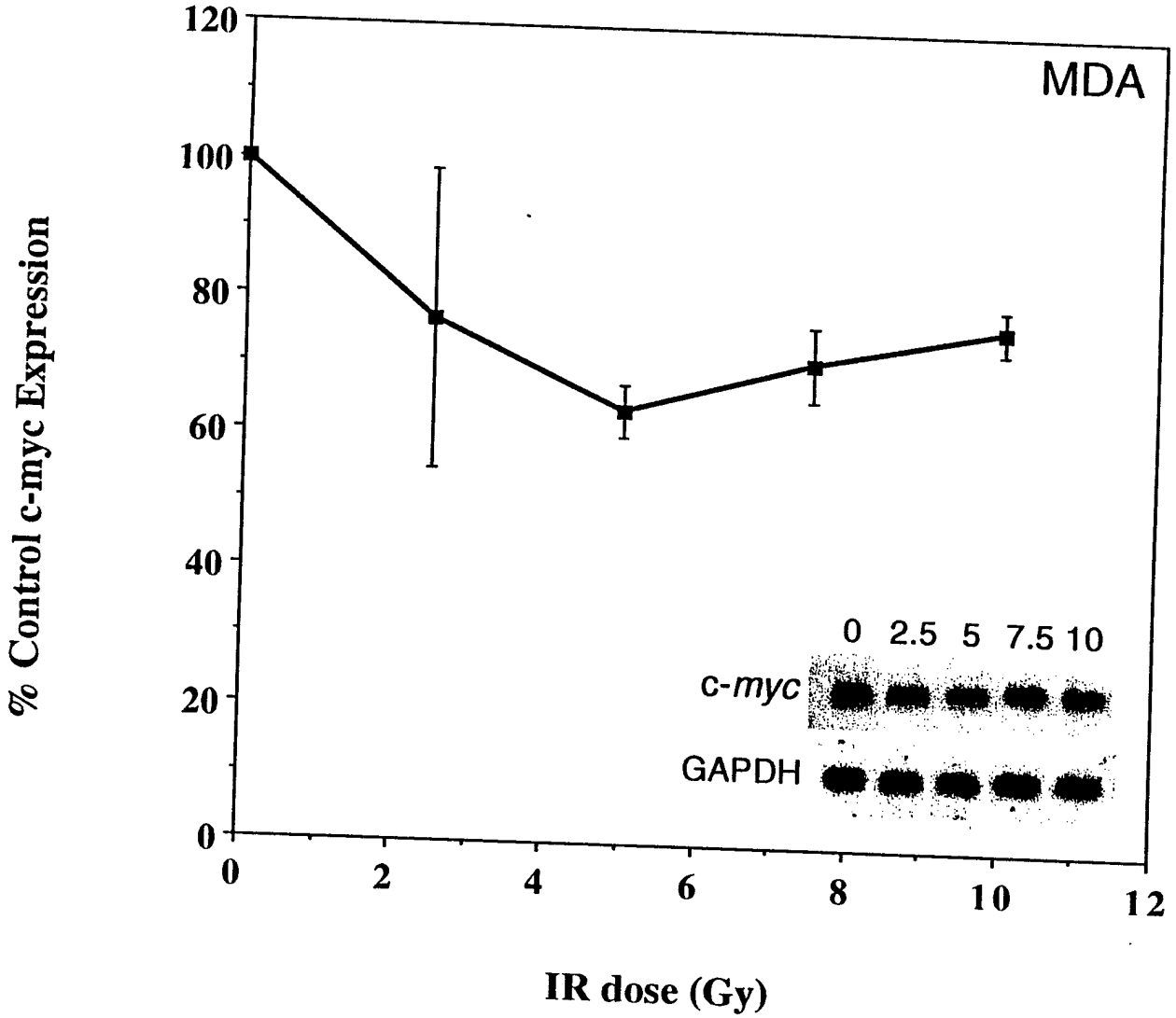

MDA
% Control c-myc Expression
IR dose (Gy)
0
20
40
60
80
100
120
0
2
4
6
8
10
12
0 2.5 5 7.5 10
c-myc
GAPDH

Figure 3

MCF-7 CONTROLS

10 Gy 48 HOURS

20 Gy 48 HOURS

MDA CONTROLS

10 Gy 48 HOURS

20 Gy 48 HOURS

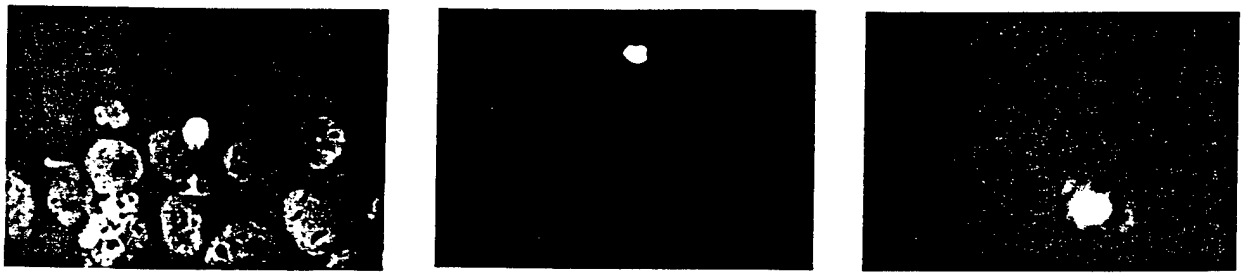


Figure 4

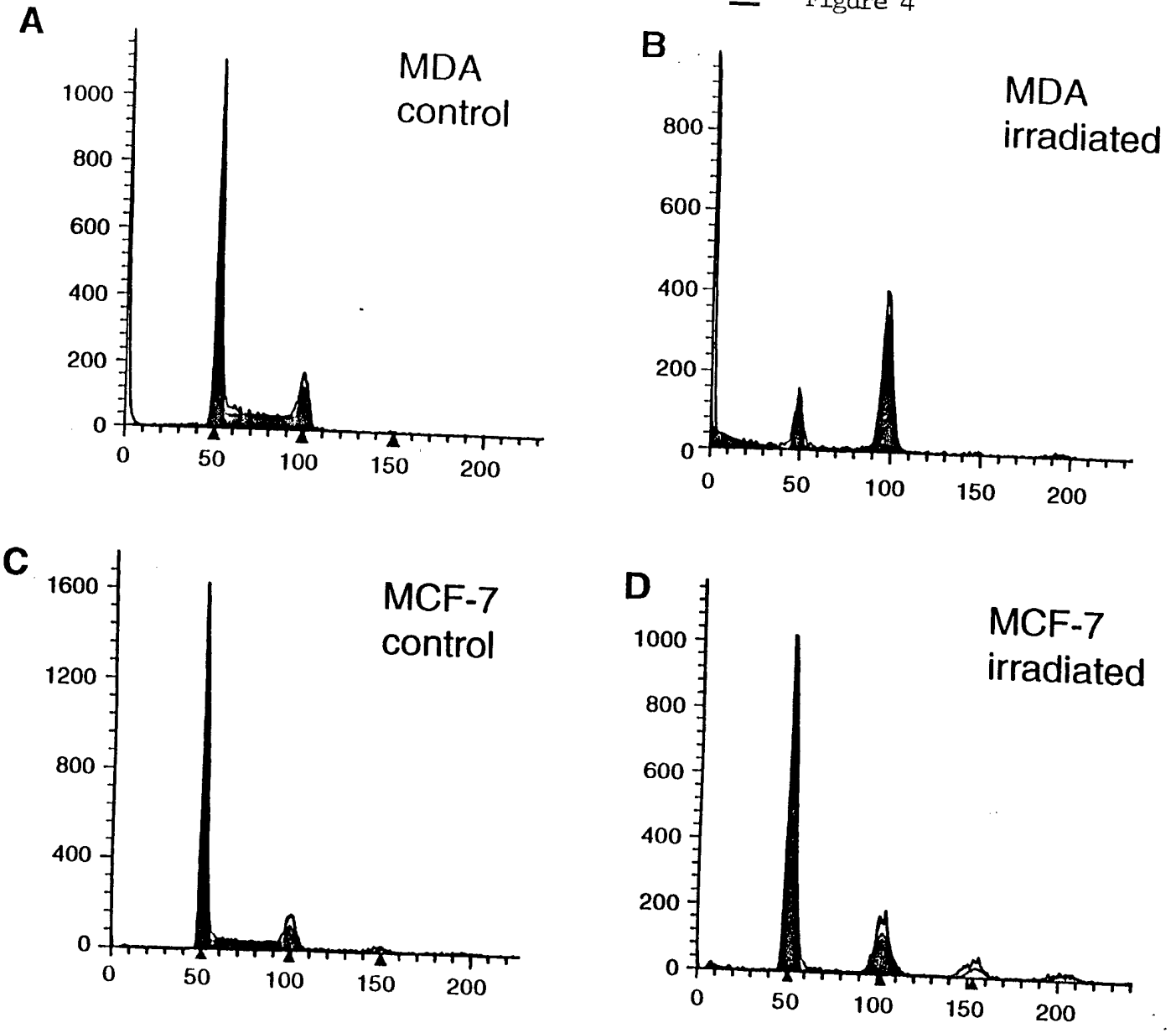

A
MDA
control
1000
800
600
400
200
0
0
50
100
150
200
B
MDA
irradiated
800
600
400
200
0
0
50
100
150
200
C
MCF-7
control
1600
1200
800
400
0
0
50
100
150
200
D
MCF-7
irradiated
1000
800
600
400
200
0
0
50
100
150
200

Figure 5

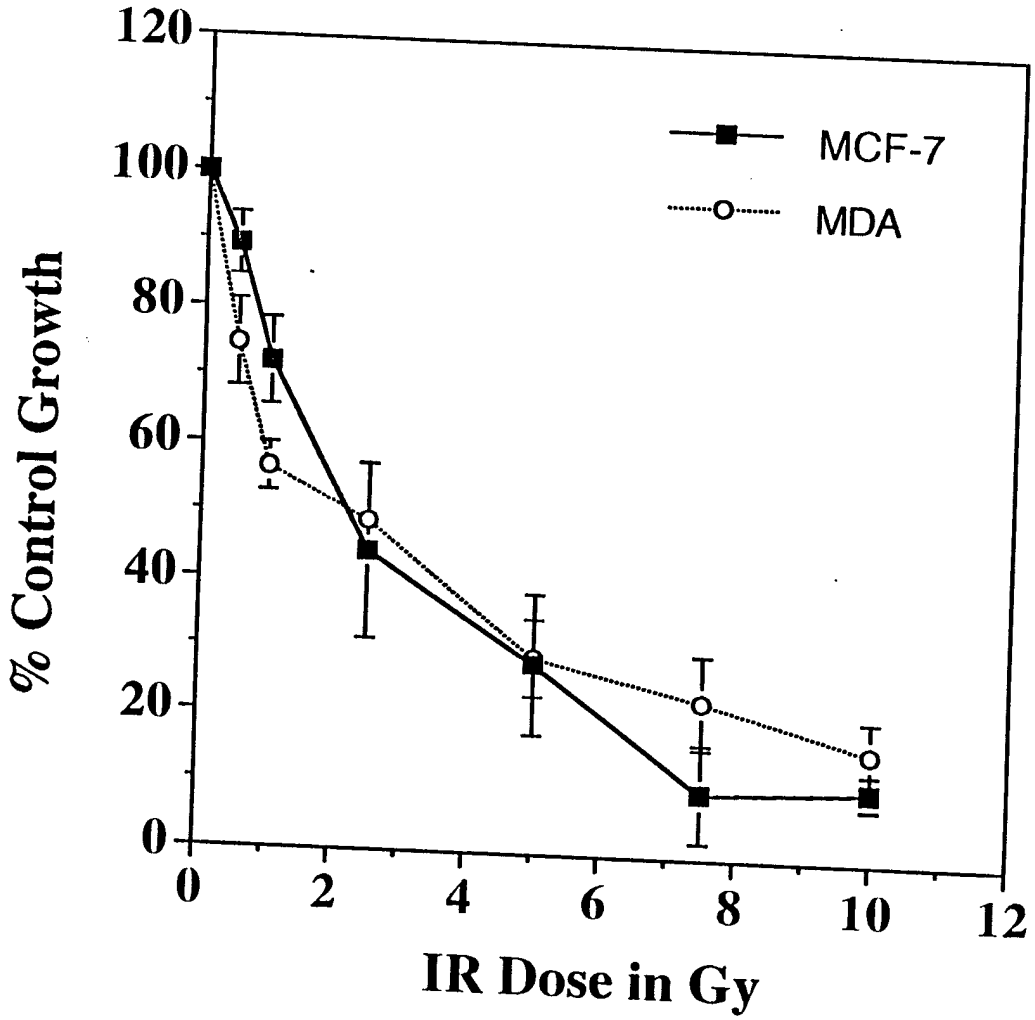

120
100
80
60
40
20
0
0
2
4
6
8
10
12
% Control Growth
IR Dose in Gy
MCF-7
MDA

Figure 6

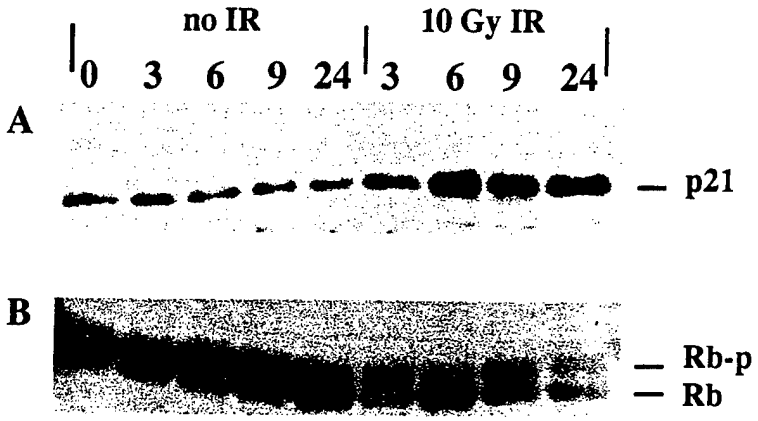
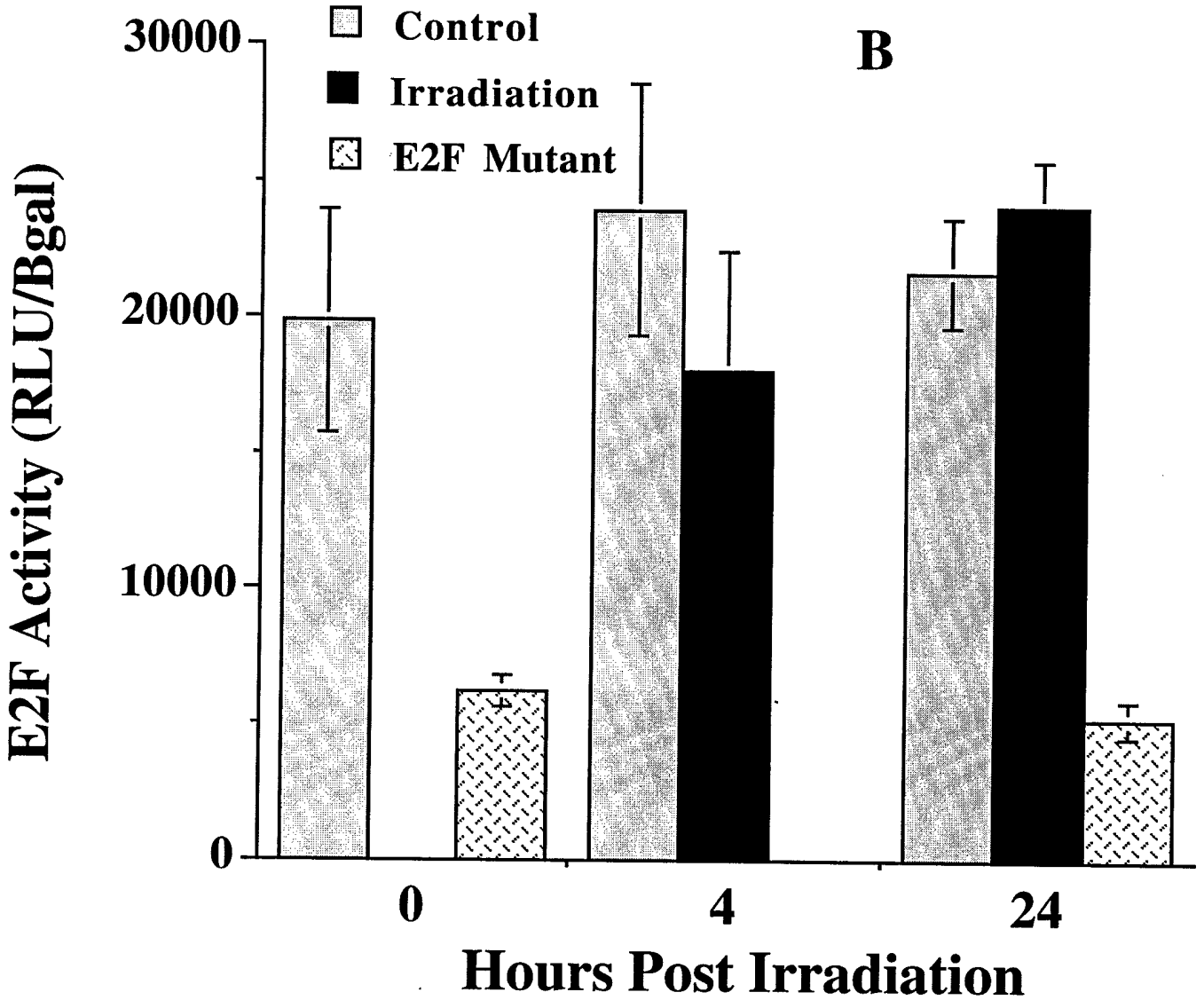
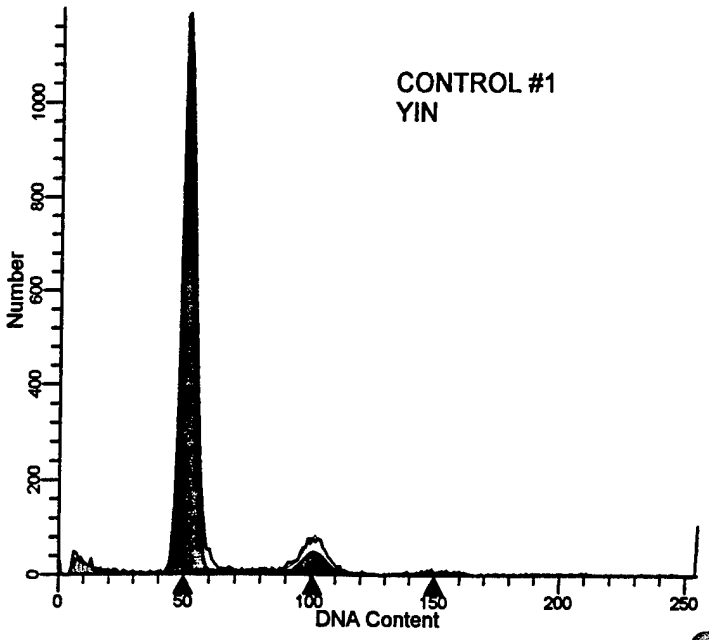


Figure 7

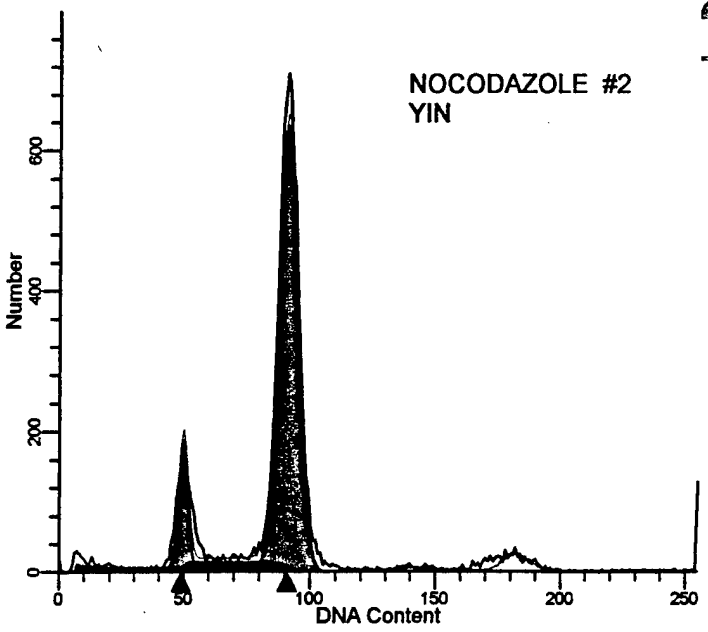

B
Control
Irradiation
E2F Mutant
E2F Activity (RLU/Bgal)
30000
20000
10000
0
0
4
24
Hours Post Irradiation



Date acquired: 29-APR-97
File: DNA296
Sample: SAMPLE ID
Sample tag: SAMPLE TAG
Analysis type: Automatic analysis
Prep: Fresh/Frozen

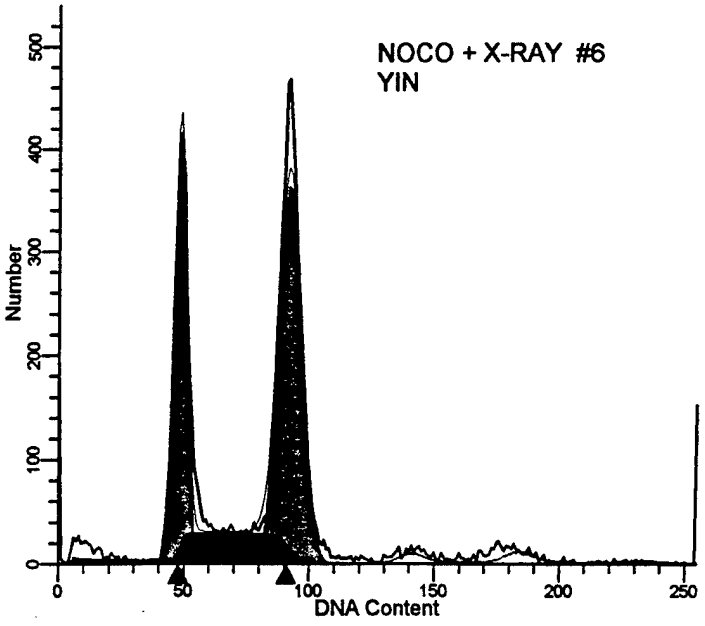
DIPLOID: 100.00 %
Dip G0-G1: 86.88 % at 51.26
Dip G2-M: 7.47 % at 101.63
Dip S: 5.66 % G2/G1: 1.98
Dip %CV: 4.94
Total S-Phase: 5.66 %
Extra Pop: %
Debris: 4.24 %
Aggregates: 5.74 %
Modeled Events: 9322.86 RCS: 2.587

Figure 8



Date acquired: 29-APR-97
File: DNA297
Sample: SAMPLE ID
Sample tag: SAMPLE TAG
Analysis type: Manual analysis
Prep: Paraffin

DIPLOID: 100.00 %
Dip G0-G1: 11.99 % at 49.83
Dip G2-M: 80.10 % at 91.36
Dip S: 7.91 % G2/G1: 1.83
Dip %CV: 3.93
Total S-Phase: 7.91 %
Extra Pop: %
Debris: 7.18 %
Aggregates: 7.00 %
Modeled Events: 9095.22 RCS: 3.684



Date acquired: 29-APR-97
File: DNA301
Sample: SAMPLE ID
Sample tag: SAMPLE TAG
Analysis type: Manual analysis
Prep: Paraffin

DIPLOID: 100.00 %
Dip G0-G1: 30.77 % at 48.68
Dip G2-M: 53.00 % at 92.32
Dip S: 16.23 % G2/G1: 1.90
Dip %CV: 4.71
Total S-Phase: 16.23 %
Extra Pop: %
Debris: 3.81 %
Aggregates: 6.55 %
Modeled Events: 8772.94 RCS: 4.848